The application of the paraffin-imbedding method in botany.

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In the following lines it is my purpose to introduce into botanical science the paraffin-imbedding method which zoologists generally have employed for several years, and with great success. It will be understood that the method here meant is that in which whole organisms, or parts of them, are so imbedded as to be entirely permeated with paraffin. In fact, if the operation has succeeded well it will be impossible to distinguish the imbedded object from the surrounding paraffin except by its color. The principal advantages of this treatment especially appear when it is combined with the more excellent methods of microscopical science: for arresting protoplasm in its living form, for section-cutting and the mounting of specimens. Thus it not only enables the observer to make sections of very minute and tender objects, but also to obtain, with the greatest ease, even in very difficult cases, sections through previously determined parts of these objects, and, moreover, rigorously in the required directions. It is also possible by these means to prepare a series of consecutive sections, and it is obvious of how much use this may be in studying the development of many organs. Lastly, it is of some consequence that in sections made after this method parts which otherwise are not united to each other may still be kept in their relative positions. Thus it is possible to make thin transverse sections of buds in which the disposition of the leaves remains unaltered, and may be studied with ease. That, notwithstanding these advantages, the imbedding method has not been made use of to any extent[1] in botany must be probably ascribed to various causes. One cause of the failure of experiments in this direction may have been owing to the fact that the imbedding method was not combined with other methods; and still, as has already been remarked, this is necessary in order to insure success. In the second place no vegetable parts preserved in alcohol should be used for imbedding, as it will be often found very difficult to permeate them with paraffin. On the contrary, it is necessary to employ chromic or picric acid,

[1] Only one botanist has, as far as I know, employed the imbedding method, viz. S. Schönland, who described the beautiful results obtained with paraffin imbedding and the rocking-microtome. Ein Beitrag zür Mikroskopischen Technik. Bot. Centralblatt, 1877, vol. 22, p. 283.

or mixtures of these with other substances. In such liquids fresh material should be kept for some time, and then only alcohol should be applied for withdrawing the water it contains previous to imbedding. I think that this peculiarity is connected with the presence of cellulose, which prevents the paraffin from permeating many vegetable organs, but is somewhat macerated by chromic or picric acid and other reagents. Thirdly, the imbedding method has, perhaps, been often tried in the case of full-grown parts, and with these, in many instances, it will not succeed so well. Sometimes it is difficult to permeate them with paraffin. In many cases, however, this can be attained, but even then sections made without previous imbedding are often to be preferred. Moreover, it will but seldom be necessary to have recourse to this method with adult organs, because with these it is in general easy enough to obtain all the sections required in the usual manner. Still there are cases in which the imbedding of full-grown parts is very useful, and in several instances I have succeeded in it very well.

My researches, however, speedily convinced me that the proper sphere for the application of the imbedding method, especially by those botanists who try it for the first time, is to be found in meristematic tissues, the cells of which contain but little cell-sap, a thin cell-wall, and much protoplasm, and in these respects may be compared to animal tissues. In these cases I have met with signal success, and it seems that this is not wholly without importance, as it is precisely with growing points of stems and roots that the advantages of imbedding are invaluable. I do not mean to assert that in this manner results are to be obtained which are absolutely not to be had by having recourse to the usual methods of preparing meristematic tissues for observation. It must be admitted that perseverance and patience have effected much in these matters. But it is certain that by this method the same and better results may be obtained with the greatest ease, which formerly were achieved only by comparatively few observers, with much exertion and loss of time. Thus every student may see many things which he otherwise would not have seen: longitudinal sections accurately through the median line of growing points, a series of consecutive transverse sections of the same objects, etc. And he may have such specimens in profusion, whilst every one who has been engaged in these researches knows that by following the usual methods one is often compelled to be con-

tent with a single section successfully accomplished. It is especially for this reason that I write these lines. I am convinced that it will prove useful when more observers are enabled to study the internal development of vegetable organs than was hitherto the case.

It is also very fortunate, that with the imbedding method the application of those reagents now generally employed for fixing protoplasm in its living form can be combined. Thus specimens are obtained in which the protoplasts retain, in a great measure, their original appearance. That this is the case will be admitted, when I mention that in cells with a large amount of cell-sap the peripheral protoplasm remains entirely united to the cell-wall: that the sections of growing points exhibit in the most beautiful manner the process of cell-division, with its several karyokinetic figures; and lastly, that even in the youngest cells vacuoles[2] may be distinctly seen. Formerly the investigator was often obliged to dissolve the protoplasmic contents of meristematic cells with caustic potash or similar reagents, in order to make their forms visible. Now this has become wholly superfluous, and the protoplasm may be observed in the cells of the tenderest meristematic tissue, whilst the contours of these cells are rendered as distinct as can be desired by employing the staining agents commonly in use.

In trying to apply the imbedding method to vegetable objects, I have followed the methods by which zoologists obtain their specimens, and I can not say that I have discovered anything essentially new. But still, some special precautions are to be taken. As everywhere else in microscopical research, it is difficult, if not impossible, to give general rules that will hold good for the treatment of all objects. On the contrary, it will, in most cases, be found necessary to treat different objects in a slightly different manner, and it will be the task of the observer to find out in each case which way he should follow.

Thus it seems most rational, instead of giving general, and therefore partially inexact directions, to describe a single instance at full length. If anybody should wish to become acquainted with the imbedding method I advise him to do precisely what I shall describe here, and he will easily succeed in obtaining the same results. He may then apply this method to other objects, which must, perhaps, be treated somewhat differently.

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[2]Went. Les premiers états des Vacuoles. Archives Neerl. 1887.

I will describe the imbedding method as employed in preparing the growing points of roots, for these are very convenient objects and have been tried by me in the most various ways. The primary roots of germinating seeds of Vicia Faba, or the secondary roots of the bulb of Allium Cepa (grown in water) should be taken for this purpose, for in these large and beautiful karyokinetic figures are with certainty to be found. In the roots of Phaseolus multiflorus, Zea Mays or Æsculus Hippocastanum, on the contrary, these figures are small and indistinct.

Fresh tips of roots, 1 or 2 centim. long, are conveyed into a sufficient quantity of a reagent calculated to arrest living protoplasm in its original form. Several substances may be employed in our case. I obtained very good results with a watery solution of chromic acid (1%), with a saturated solution of picric acid, etc.; but absolute alcohol must not be applied, as not only the roots are totally shriveled in this fluid, but moreover, because, as has already been observed, it is often difficult to permeate objects thus treated with paraffin. The most beautiful specimens, however, were obtained from roots which had been immersed for some time in Flemming's Mixture slightly altered. We will suppose that such a liquid is used, viz.: a watery solution containing chromic acid 1%, osmic acid 0.02%, and acetic acid 0.1%. Mixtures containing more osmic acid are to be preferred, especially if the karyokinetic figures are to be studied. As, however, a larger amount of osmic acid or the same amount combined with less chromic acid is apt to cause some difficulty in the imbedding, at least for beginners I advise to employ the mixture here recommended. If the karyokinesis is to be studied, it will be advisable to remove on both sides of the root-tip a slice of tissue in order to facilitate the entrance of the reagents.

In this mixture the root-tips remain from twenty-four to forty-eight hours, and then, the protoplasm being fixed, the acids are washed out in running water. For this purpose they are put into a vessel, with a double perforated cork stopper, holding a funnel for receiving a jet of water, and in the second orifice a reversed U-tube, with one leg reaching to the bottom of the flagon, so that it acts as a syphon[3]. By these means the roots are kept for five or six hours in a continual stream of fresh water, and after this time it may be confidently asserted that the acids have been thoroughly

[3]Suggested by Prof. Pekelharing.

washed out of them[4]. Then the roots must be put into alcohol, in order to replace the water they contain by this reagent. This manipulation, however, must be conducted with great caution, because the root-tips are very liable to be shrivelled.

I have no doubt that Schulze's apparatus[5] may be used here with success, but I found that it is sufficient to bring the roots, successively for some hours, or half a day, into alcohol of 20%, 40%, 60%, 80% ,95%, and finally into absolute alcohol. By these means shriveling may be totally avoided; whilst the manipulations are very simple, when bottles, containing alcohol of the concentrations required, are always kept ready and renewed from time to time.

Now, the alcohol should be replaced by a solvent of paraffin, for instance chloroform, benzol or turpentine. These fluids may be employed promiscuously, but I prefer turpentine as being the least volatile of the three. The roots are first brought into a mixture of absolute alcohol and turpentine in equal parts, and after some hours into pure turpentine, and this again in order to avoid shriveling. After some hours the roots may be put into a cold saturated solution of paraffin in turpentine. From thence the roots are removed into a mixture of equal parts of turpentine and paraffin, kept at a constant temperature of from 30 to 40° C, in an ordinary drying oven furnished with a gas regulator. After having remained an hour in this liquid the temperature is raised to from 50 to 55° C, and the roots are finally placed in pure melted paraffin, which is renewed one or two times.

I generally prefer a tolerably firm paraffin, which melts at a temperature of about 50° C. When the root-tips have remained six or eight hours in this condition one may be sure that they are wholly permeated with paraffin, and are ready for use. They now must be placed in a block of paraffin of a regular form, which may easily be held in the microtome, and they should be so placed in it as to enable the observer to make sections in the direction required. For this purpose I employ a well-known arrangement, consisting of a flat metal plate, on which are laid two Gothic-shaped pieces of metal, against each other, so as to form a rectangular mould for receiving the paraffin. I generally employ these Gothic-shaped pieces of two sizes, according to the form of the objects to be imbedded. One pair has legs of 1.5 and 4.5,

[4]Roots from picric acid should be washed in alcohol of from 20 to 40 per cent.

[5]Archiv. für mikr. Anat. Bd. 25, p. 542.

another of 2.2 and 5 centimeters. Both have a height of one centimeter. The metal pieces and plate are slightly wetted with turpentine, to prevent the paraffin from adhering to their surface, and then melted paraffin is poured into the rectangular space till it is nearly filled. The root-tips have all this while remained in the drying-stove, but now they are taken out and put into the paraffin filling the mould. If this was somewhat over-heated when the operation began, and needles heated in the flame are employed, there will be ample time for arranging the root-tips in the directions required before the paraffin cools. The objects will not stick to the bottom of the mould, because the paraffin here hardens almost instantaneously on coming into contact with the cold surface of the metal. As soon as the molten mass has cooled so far as to be covered with a thin film on its upper surface, cold water should at once be poured over it, for which purpose the whole apparatus is placed from the beginning in a flat basin.

This sudden hardening of the paraffin serves to prevent the formation of cavities in it, which otherwise will sometimes occur and make it impossible to obtain good sections. We now proceed to the section-cutting, which should be performed in this case with a microtome. Thus only can the advantages of the imbedding method be fully enjoyed, and especially if a series of consecutive sections is made. This, if transverse, will most beautifully exhibit the whole process of development going on in the root-tip, and if longitudinal it will be very easy and save much labor to select from the series one or a few sections which have passed through the median line of the root. Though greatly preferring the arrangement of the microtomes of the Cambridge scientific instrument company, I selected an instrument constructed on less exact principles, Caldwell's microtome being beyond my means, and the so-called rocking microtome not allowing the object to be moved in the three directions of space. This, however, is often necessary in making sections of roots and other vegetable organs, especially if growing points are to be examined. I was often compelled slightly to alter the direction of the root, even at the very last moment, particularly when cutting longitudinal sections[6]. I therefore employed a microtome of Schanze, which, though in several respects a defective instrument, has not the draw-

[6] Of late, however, I have become more and more convinced that with some practice it would be possible to employ the rocking microtome, and Dr. Schönland's experience (l. c.) has greatly strengthened this conviction.

back mentioned above. It should be observed, however, that this microtome is properly not adapted for making a series of sections, as the knife can not be placed in a transverse position. But this difficulty may be overcome by reversing that part which holds the object and further making some slight alteration in its arrangement, by which, however, the instrument loses still more of its stability, already not very great. Notwithstanding these objections, I succeeded in making ribands of sections well enough, if needed, keeping them flat with a piece of cross-wire held in the left hand while cutting with the right. The sections thus gained must now be glued to the slide before dissolving the paraffin. If a series of sections has been made, or sections in which parts not united to each other should be kept in their relative positions, the necessity of this method is obvious. But in almost all other cases it deserves to be applied, as it facilitates in a great measure the following manipulations of staining and mounting, and thereby enables the observer to prepare, with little loss of time, a great number of specimens. From these he may then choose at leisure the most successful ones, or those exhibiting the phenomenon sought for, and reject the rest.

The operation of gluing the sections is very simple, and may be performed in several well-known ways. As far as I know, it is best to employ a solution of india rubber, albumen or collodion. I mostly used the last two substances. When albumen is employed for this purpose the white of an egg is clipped with a pair of a scissors, the same volume of glycerine is mixed up with it, and after adding some drops of carbolic acid and filtering it is fit for use.

If collodion should be preferred, it suffices to mix equal quantities of this substance and oil of cloves. Both these mixtures do almost equally well, and are applied in exactly the same manner. A very thin layer of the fluid is spread with a camel's-hair pencil over that part of the slide (or the cover-glass) to which the sections are to be stuck. Then the paraffin sections are laid in their places and gently pressed against the slide with the brush or with the finger. After this the slides remain for a quarter of an hour in the oven, at a temperature of about 50° C. There the paraffin melts and the sections settle into the layer of gluing substance. It is also sufficient to heat the slides cautiously for one or two minutes over the flame. In both cases the slides, when still warm, are plunged into

turpentine, which soon entirely dissolves the paraffin. The sections now stick firmly to the glass and the slides can pass through various liquids without the sections being detached. After having remained for some time in turpentine, this is washed out with alcohol of 95 %.

We now proceed to staining the sections, but I can not enter into many details here, which are to be sought for in works treating of microscopic manipulation.

I only observe that sections made in the manner here described require to be stained in order fully to enjoy the advantages of the imbedding method.

I will add some directions as to the staining of the specimens which we have here chosen as an example, viz., the roots of Vicia or Allium. These roots may be stained before imbedding, but in this case it will be preferable to use roots which have been treated with picric or chromic acid instead of Flemming's Mixture.

I put them for twenty-four hours into a solution of Grenacher's alum-carmine after they have reached the alcohol of 60%. After this they come again into alcohol and are further treated as described above. When the paraffin has been dissolved out of the sections by means of turpentine, it may be directly replaced in this case by a mounting medium: oil of cloves, Canada balsam or glycerine, the latter after having replaced the turpentine by alcohol.

In most cases, however, it will be preferable to proceed to staining only after the sections have been prepared as above, especially if specimens have been made in order to try the imbedding method. In the case of a single root many slides may be obtained, and thus it will be easy to try the effect of various staining reagents.

If only a general survey of the internal structure of the growing point is intended, I recommend the employment of alum-carmine, in which the slides should remain from twelve to twenty-four hours. With hæmatoxylin a similar effect is obtained, and if this should be applied at a temperature of 50° C. the process of staining will only take from ten to twenty minutes.[7] With both these dyes the protoplasm,

[7] I insert here a formula for obtaining in a few hours a solution of hæmatoxylin which will remain fit for use for a very long time without forming a precipitate. As it is given in a dissertation, written in the Dutch language, on a subject of pathological anatomy, it will not, perhaps, come to most botanists' notice: Three parts of hæmatoxylin are dissolved in two parts of absolute alcohol. Five parts of this solution are added, drop by drop, to 100 parts of a 3 per cent. aqueous solution of alum. This fluid is kept for two hours in a covered but not hermetically closed glass vessel at a temperature of 40° C., and after having been filtered it is immediately fit for use. Some carbolic acid is added to it, and for staining it is used ten times diluted with water.—*D. G. Siegenbeek von Heukelom, Pathologisch bindweefsel*, 1885.

and especially the nuclei, are beautifully colored, and the colors of the cells will be plainly discernible, so that it will be a very easy matter to make an exact drawing of the whole growing point.

For mounting these specimens glycerine or Canada balsam may be used indifferently, but I generally prefer the latter because the coloring matter is better preserved in it.

If, however, the observer wishes to see the karyokinetic figures with which the meristematic tissue of the root tip abounds, it will be necessary to have recourse to the coal-tar colors. With the roots already repeatedly mentioned, I obtained beautiful results in the following manner:

The slides, with the sections glued to them, are taken from the alcohol, where we left them last. They are washed for some moments in pure water and then placed in a watery solution of gentian-violet (Trommsdorff), which is procured by adding 1 part of a saturated alcoholic solution of the dye to 1000 parts of water. Here they remain for six to twenty-four hours, or at a temperature of 50° C. for a much shorter time (one hour). After this they are treated for some seconds with absolute alcohol, containing $\frac{1}{8}\%$ or less of hydrochloric acid, then washed out well, first in water with a few drops of ammonia, (e. g. 10 drops in 300 cub. cent. of water), afterwards in neutral alcohol. Finally, the sections are mounted in oil of cloves, and afterwards in Canada balsam. Successful preparations of this kind will exhibit most beautifully the longitudinal division of the segments into which the nucleus is dissolved.

Similar, but not quite as beautiful, results are to be obtained with safranin if employed in the same manner as gentian-violet, or as Dr. H. Twaardemaher recommends:[8] To a concentrated watery solution of anilin-oil is added an equal quantity of a concentrated alcoholic solution of safranin. In this liquid the slides remain for one hour, then they are washed for a very short time in acid alcohol, and treated as above, after staining with gentian-violet. I am fully convinced that anybody who should try to prepare specimens of root-tips after the methods here described will easily obtain the same results, and if he has once seen them he will certainly apply the imbedding method in many other cases, and find it very useful. If any of my fellow-botanists should wish to obtain a notion of the results to be gained before trying the method themselves, I shall

[8] Maandblad voor Natuurwetenschappen. T. 14, 1887, p. 6.

be very happy, on being applied to (Nachtegaalstraat 32), to send them a specimen. It is no doubt an inconvenience of this method that the observer must wait for some days before he can make his sections. This, however, is no serious objection, as the operations described take up but very little of his time, so that he may do other work whilst his objects are getting ready for imbedding. Moreover, if many objects are to be treated in this manner, it will be easy, by a regular distribution of labor, to have always material ready for examination. Other objects fit for trying the imbedding method on are the growing points of the stems of Vicia Faba, Elodea Canadensis, Æsculus Hippocastanum, Acer pseudoplatanus, Equisetum, etc. I also obtained very beautiful specimens by making longitudinal and transverse sections of whole plants of Mnium hornum, the first showing the antheridia and archegonia, the second showing very plainly the disposition of the leaves and their development.

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BRIEFER ARTICLES.

Some results of mycological work in U. S. Dept. of Agriculture.—Among the discoveries of botanical interest as well as practical importance made through the efforts of the commissioner of agriculture the past season, the following may be mentioned:

1st. That of Greeneria (*G. fuliginea* Scribner & Viala), the fungus which causes what grape-growers term "bitter rot." The studies of this fungus were begun in the vineyard of Hon. Wharton J. Green, of Fayetteville, N. C., whose courtesy enabled very careful observations to be made upon its external appearance and effects, and, in constituting a new genus upon the species discovered, it was a pleasure to the authors to name it for Mr. Green as a mark of their esteem and respect. Later observations showed this fungus to be widely distributed over the country east of the Mississippi and westward to Texas. Under special conditions it does much damage to the crop, attacking the berries during the period of ripening.

2d. That of *Coniotherium diplodiella* Sacc., which was first discovered in Italy, in 1879, by M. Spegazzini. In 1885 it was observed for the first time in France. Its distribution in this country is not known, but its present limits seem to be southwestern Missouri and northeastern Indian Territory, where it was observed for the first time the present season. Its effect on the berries has led to the use of the term "white rot" for this disease. In France, the past season, it has occasioned considerable alarm on account of the extent of its ravages.